

REMARKS

CLAIM AMENDMENTS

Claims 1, 2, 10-29, 35, 46-49, 57, 58, and 78-83 are pending. Claims 3-9, 30-34, 36-45, 50-56, and 59-77 were previously withdrawn from consideration pursuant to a restriction requirement. In the Office Action dated August 30, 2004, the Office indicated claims 2, 27, 58, 78 and 79 were allowed.

The proposed amendments are as follows:

- (a) The cancellation without prejudice of claims 1, 10-26, 28-29, 35, 46-49 and 57; and
- (b) The addition of new claims 84-110.

Upon entry of the proposed amendments, claims 2, 27, 58, 78-79 and 84-107 will be pending in the application. The pending independent claims will be claims 2, 27, 58, 78, 79, 90, 99, 103, 104, 107 and 108.

The proposed amendments to claims 2, 27, 58, 78, and 79 are clarifying amendments. No new matter has been added. The amended claims are supported by the specification and by the claims as originally filed. More specifically, claim 27 has been amended from its original form as a claim depending from (canceled) claim 17 to an independent claim. Support for this amendment is found in the originally filed claims 15, 16, and 17. Similarly, Claim 58 has been amended from its original form as a claim depending from claim 2 to an independent claim. Support for this amendment is found in originally filed claims 2 and 58.

New claims 84-110 are presented in this Amendment C. Support for these claims is found in the specification as provided, for example, in the table below. Throughout these

remarks, and as discussed with the Examiner on October 27, 2004, Applicants refer to page numbers presented in the center of the header of each page of this application.

<u>Current Claim #</u>	<u>Supporting Citation(s)</u>
84	(Depends from 78) <ul style="list-style-type: none"> pg. 12, lines 3-17: discuss the use of SEQ ID NO 2 and variants thereof that are at least 95% identical Originally filed claim 10 pg. 57, Example 11: SEQ ID NO 1 is isolated from <i>A. ochraceus</i> describes cloning the polypeptide from <i>A. ochraceus</i> cultures pg. 67-69, Example 20: data described (presented in Figure 16) shows the cloned polypeptide catalyzed the 11 alpha hydroxylation of androstenedione pg. 19, structure #2: canrenone has both a 3-keto and a 4,5-double bond pg. 20, structure #6: androstenedione has both a 3-keto and a 4,5-double bond
85	(Depends from 84) Selection of androstenedione from new claim 84
86	(Depends from 84) Selection of canrenone from new claim 84
87	(Depends from 79) <ul style="list-style-type: none"> pg. 12, lines 3-17: discuss the use of SEQ ID NO 2 and variants thereof that are at least 95% identical Originally filed claim 10 pg. 57, Example 11: SEQ ID NO 1 is isolated from <i>A. ochraceus</i> describes cloning the polypeptide from <i>A. ochraceus</i> cultures pg. 67-69, Example 20: data described (presented in Figure 16) shows the cloned polypeptide catalyzed the 11 alpha hydroxylation of androstenedione pg. 19, structure #2: canrenone has both a 3-keto and a 4,5-double bond pg. 20, structure #6: androstenedione has both a 3-keto and a 4,5-double bond
88	(Depends from 87) Selection of androstenedione from new claim 87
89	(Depends from 87) Selection of canrenone from new claim 87
90	<ul style="list-style-type: none"> Originally filed claim 10 pg. 57, Example 11: SEQ ID NO 1 is isolated from <i>A. ochraceus</i> describes cloning the polypeptide from <i>A. ochraceus</i> cultures pg. 67-69, Example 20: data described (presented in Figure 16) shows the cloned polypeptide catalyzed the 11 alpha hydroxylation of androstenedione pg. 19, structure #2: canrenone has both a 3-keto and a 4,5-double bond pg. 20, structure #6: androstenedione has both a 3-keto and a 4,5-double bond
91	(Depends from 90) <ul style="list-style-type: none"> pg. 6, lines 24-33: polypeptide of SEQ ID NO 2 ... with an amino acid sequence of at least ... 50%
92	(Depends from 90)

	<ul style="list-style-type: none"> pg. 6, lines 24-33: polypeptide of SEQ ID NO 2 ... with an amino acid sequence of at least ... 75%
93	(Depends from 90) Selection of androstenedione from new claim 90
94	(Depends from 93) <ul style="list-style-type: none"> pg. 6, lines 24-33: polypeptide of SEQ ID NO 2 ... with an amino acid sequence of at least ... 50%
95	(Depends from 93) <ul style="list-style-type: none"> pg. 6, lines 24-33: polypeptide of SEQ ID NO 2 ... with an amino acid sequence of at least ... 75%
96	(Depends from 90) Selection of canrenone from new claim 90
97	(Depends from 96) <ul style="list-style-type: none"> pg. 6, lines 24-33: polypeptide of SEQ ID NO 2 ... with an amino acid sequence of at least ... 50%
98	(Depends from 96) <ul style="list-style-type: none"> pg. 6, lines 24-33: polypeptide of SEQ ID NO 2 ... with an amino acid sequence of at least ... 75%
99	<ul style="list-style-type: none"> Originally filed claim 15 pg. 35, lines 17-29: several methods for producing polypeptides of the present invention pg. 64-65, example 16, lines 19 – 28: transfection of insect host cells using baculovirus shuttle vector pg. 35-37: generally expression vectors, with many art citations pg. 38-41: generally transformation and transfection pg. 39-41: generally insect cell expression - using strong baculovirus promoter (pg. 40, line 20) – polyhedron or p10 promoter pg. 40, lines 8-15: Pure recombinant baculovirus carrying the 11 alpha hydroxylase are used to infect cells. pg. 40, lines 24-28: expression by use of promoters in insect cells
100	(Depends from 99) Selection of androstenedione from new claim 99
101	(Depends from 99) Selection of canrenone from new claim 99
102	(Depends from 99) <ul style="list-style-type: none"> Originally filed claims 15, 16, 17 and 27 pg. 57-58, example 11: cloning of 11 alpha hydroxylase, resulting in SEQ ID NO:1 (nucleic acid), SEQ ID NO:2 (protein) after amplification and analysis (pg. 58, lines 14-16) pg. 64 – example 16, insect infection and expression
103	<ul style="list-style-type: none"> Originally filed claims 2 and 35 pg. 35-37: general information on expression vectors and how to make them, in yeast and mammalian systems. pg. 43-44: table containing different plasmids for gene expression, specifically pg. 43: pMON45624 for 11 alpha hydroxylase
104	(Depends from 103)

	<ul style="list-style-type: none"> pg. 11, lines 11-12: describe host cell and at least one expression cassette pg. 35-37: expression vectors generally
105	(Depends from 104) <ul style="list-style-type: none"> pg. 11, lines 25-27: expression cassette into a chromosome of host cell
106	(Depends from 104) <ul style="list-style-type: none"> pg. 11, lines 25-27: expression cassette into an expression vector
107	<ul style="list-style-type: none"> Originally filed claims 10 and 35 pg. 35-37: general information on expression vectors and how to make them, in yeast and mammalian systems. pg. 43-44: table containing different plasmids for gene expression, specifically pg. 43: pMON45624 for 11 alpha hydroxylase
108	(Depends from 107) <ul style="list-style-type: none"> pg. 11, lines 11-12: describe host cell and at least one expression cassette pg. 35-37: expression vectors generally
109	(Depends from 108) <ul style="list-style-type: none"> pg. 11, lines 25-27: expression cassette into a chromosome of host cell
110	(Depends from 108) <ul style="list-style-type: none"> pg. 11, lines 25-27: expression cassette into an expression vector

CLAIM REJECTIONS UNDER 35 U.S.C. § 112, FIRST PARAGRAPH

The Office rejected Claims 10, 12-13, 15-26, 28-29, 35, 46-49, and 80-83 under 35 U.S.C. §112, first paragraph asserting that the specification, while being enabling for nucleic acids comprising SEQ ID NO:1 and nucleic acids that hybridize under high stringency conditions to SEQ ID NO:1, does not reasonably provide enablement for claims of the scope of the instant claims. The rejection is respectfully traversed.

Applicants assert that the claims are fully enabled. For example, the specification clearly teaches the following:

(1) general techniques for isolating a nucleic acid encoding an 11-alpha hydroxylase from *Aspergillus ochraceus* (see, e.g., Examples 1-11 of the specification).

(2) the use of the 11-alpha hydroxylase encoded by the isolated nucleic acid to selectively catalyze the 11-alpha hydroxylation of a steroid compound comprising the following structural

features: a keto group at the 3 position (3-keto substituent) and a double bond between carbons located at the 4 and 5 position (4,5-double bond) (see page 7, lines 13-34, of the specification, which lists several classes of substrate steroids along with representative species).

(3) the use of the 11-alpha hydroxylase encoded by the isolated nucleic acid to selectively catalyze the 11-alpha hydroxylation of androstenedione. The structure of the steroid androstenedione (a steroid comprising a 3-keto substituent and a 4,5-double bond) is shown as structure #6 on page 20 of the specification. Example 20 specifically describes the conversion of androstenedione to 11-alpha hydroxyandrostenedione (see page 67, line 15 through page 69, line 8, of the specification) using the expressed 11-alpha hydroxylase. Specific data associated with this conversion are presented in Figure 16.

(4) the use of the 11-alpha hydroxylase encoded by the isolated nucleic acid to selectively catalyze the 11-alpha hydroxylation of canrenone. The structure of canrenone (another steroid comprising a 3-keto substituent and a 4,5-double bond) is shown as structure #2 on page 19 of the specification. Applicants specifically state that a preferred embodiment of the invention is the conversion of canrenone to 11-alpha hydroxycanrenone using the expressed 11-alpha hydroxylase (see page 7, lines 34-35, of the specification).

(5) Example 20 is demonstrative and intended to show the use of the 11-alpha hydroxylase, not on a single substrate, but on a general class of steroid substrates comprising a 3-keto substituent and a 4,5-double bond. As indicated on page 52, lines 7-9, of the specification: "The following examples will illustrate the invention in greater detail, although it will be understood that the invention is not limited to these specific examples."

In addition, Applicants are entitled to rely upon the disclosure of WO 98/25948, a PCT application contained in the published literature before to the priority date of the pending application. WO 98/25948 (which shares common ownership with the pending application) discusses the use of *Aspergillus ochraceus* cultures to catalyze the 11-alpha hydroxylation of both androstenedione and canrenone. Example 18 describes the conversion of androstenedone to 11-alpha hydroxyandrostenedione by *Aspergillus ochraceus* (WO 98/25948, page 223, line 9 through page 225, line 5). Similarly, Example 4 describes the conversion of canrenone to 11-alpha hydroxycanrenone by *Aspergillus ochraceus* (WO 98/25948, page 179, line 4 through page 184, line 27). Pages 179-184 and 223-225 of WO 98/25948 discussing Examples 4 and 18, respectively, mentioned above are attached as Exhibit A. While WO 98/25948 does not disclose the isolated DNA or expressed polypeptide of the present application, it does clearly teach that the *Aspergillus ochraceus* microorganism itself can be used to selectively catalyze the 11-alpha hydroxylation of, *inter alia*, androstenedione and canrenone. Accordingly, Applicants maintain that the specification provides considerable guidance and direction and is clearly enabling. Withdrawal of the enablement rejection is respectfully requested.

The Examiner further indicates that one of ordinary skill in the art would not be taught by the instant specification to make and use nucleic acids of the scope of the instant claims without undue experimentation. This rejection is respectfully traversed.

The fact that experimentation is complex does not necessarily make it undue. (MPEP §2164.01). The working examples of the specification, culminating in the data presented in Figure 16, demonstrate in a stepwise fashion, to one of skill in the art, how the invention may be practiced. Further, the protocols to make and use such nucleic acids are described in the prior art; for example,

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in Sambrook et al., Molecular Cloning, A Laboratory Manual; Cold Spring Harbor Press, Cold Spring Harbor, New York (1989). Accordingly, Applicants maintain that the specification provides considerable guidance and direction and is clearly enabling. Withdrawal of the rejection based on undue experimentation is respectfully requested.

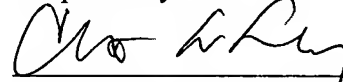
CONCLUSION

Applicants believe that the proposed amendments overcome all rejections and the claims are in condition for allowance. Thus, favorable consideration of this amendment is respectfully requested. If the Examiner believes that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at (314) 274-7008 at the Examiner's convenience.

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This paper is submitted within the 3 month shortened statutory period set for reply, accordingly no fee for extension of time is believed payable. No excess claim fees are believed payable in respect of the present amendment. In the event that an Extension of Time is required to render this paper timely filed, Applicant petitions the Commissioner under 37 C.F.R. §1.126(a) for an Extension of Time to respond for the period of time sufficient to render the paper transmitted herewith timely. The Commissioner is hereby authorized to charge any appropriate fees for filing this Amendment and any necessary Extension of Time to Deposit Account 19-1025. Further, if there is any other fee deficiency or overpayment of any fees in connection with this patent application, the Commissioner is hereby authorized to charge such deficiency or credit such overpayment to Deposit Account 19-1025.

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<u>Thielavia terricolan</u> ATCC 13807			n
<u>Trichoderma viride</u> ATCC 26802	n		
<u>Trichothecium roseum</u> ATCC 12543	tr	y	y/n
<u>Verticillium theobromae</u> ATCC 12474	y	tr	tr

Example 18

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Various cultures were tested for effectiveness in the bioconversion of androstendione to 11 α -hydroxyandrostendione according to the methods generally described above.

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A working cell bank of each of Aspergillus ochraceus NRRL 405 (ATCC 18500); Aspergillus niger ATCC 11394; Aspergillus nidulans ATCC 11267; Rhizopus oryzae ATCC 11145; Rhizopus stolonifer ATCC 6227b; Trichothecium roseum ATCC 12519 and ATCC 8685 was prepared essentially in the manner described in Example 4. Growth medium (50 ml) having the composition set forth in Table 18 was inoculated with a suspension of spores (1 ml) from the working cell bank and placed in an incubator. A seed culture was prepared in the incubator by fermentation at 26°C for about 20 hours. The incubator was agitated at a rate of 200 rpm.

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Aliquots (2 ml) of the seed culture of each microorganism were used to inoculate transformation flasks containing the growth medium (30 ml) of Table 15. Each culture was used for inoculation of two flasks, a total of 16. Androstendione (300 mg) was dissolved in methanol (6 ml) at 36°C, and a 0.5 ml aliquot of this solution was introduced into each of the flasks. Bioconversion was carried out generally under the conditions described in Example 6 for 48 hours. After 48 hours samples of the broth were pooled and extracted with ethyl acetate as in Example 17. The ethyl acetate was

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concentrated by evaporation, and samples were analyzed by thin layer chromatography to determine whether a product having a chromatographic mobility similar to that of 11 α -hydroxy-androstendione standard (Sigma Chemical Co., St. Louis) was present. The results are shown in Table 36. Positive results are indicated as "+".

TABLE 36			
Bioconversion of androstendione to 11 alpha-hydroxy-androstendione			
10	Culture	ATTC#	media TLC results
	Rhizopus oryzae	11145	CSL +
	Rhizopus stolonifer	6227b	CSL +
	Aspergillus nidulans	11267	CSL +
	Aspergillus niger	11394	CSL +
15	Aspergillus ochraceus	NRRL 405	CSL +
	Aspergillus ochraceus	18500	CSL +
	Trichothecium roseum	12519	CSL +
	Trichothecium roseum	8685	CSL +

The data in Table 36 demonstrate that each of listed cultures was capable of producing a compound from androstendione having the same Rf value as that of the 11 α -hydroxyandrostendione standard.

Aspergillus ochraceus NRRL 405 (ATCC 18500) was retested by the same procedure described above, and the culture products were isolated and purified by normal phase silica gel column chromatography using methanol as the solvent. Fractions were analyzed by thin layer chromatography. TLC plates were Whatman K6F silica gel

60Å, 10x20 size, 250μ thickness. The solvent mixture was chloroform:methanol, 95:5, v/v. The crystallized product and 11α-hydroxyandrostendione standard were both analyzed by LC-MS and NMR spectroscopy. Both compounds yielded
5 similar profiles and molecular weights.

Example 19A

Various microorganisms were tested for effectiveness in the bioconversion of androstendione to 11β-hydroxyandrostendione essentially by the methods
10 described above in Examples 17 and 18.

Cultures of each of Aspergillus fumigatus ATCC 26934, Aspergillus niger ATCC 16888 and ATCC 26693, Epicoccum oryzae ATCC 7156, Curvularia lunata ATCC 12017, Cunninghamella blakesleeana ATCC 8688a, and Pithomyces
15 atro-olivaceus IFO 6651 were grown essentially in the manner described in Example 17. Growth and fermentation media (30 ml) had the composition shown in Table 34.

The 11β-hydroxylation of androstendione by the above-listed microorganisms was analyzed using
20 essentially the same methods of product identification described in Examples 17 and 18. The results are set forth in Table 19A-1.

Table 19A-1

11β-Hydroxylation of Androstendione
25 by Various Microorganisms

	<u>Organism</u>	<u>TLC</u>	<u>LC/MS</u>
	<u>Aspergillus fumigatus</u> ATCC 26934	+	+
30	<u>Aspergillus niger</u> ATCC 16888 and ATCC 26693	+	+
	<u>Epicoccum oryzae</u>	+	+



filter. The product was dried under vacuum for 16 hours at 50°C. Yield of 11 α -hydroxycanrenone was 14 kg.

Example 4

Lyophilized spores of Aspergillus ochraceus

- 5 NRRL 405 were suspended in a corn steep liquor growth medium (2 ml) having the composition set forth in Table 15:

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TABLE 15 - Corn Steep Liquor Medium (Growth Medium for Primary Seed Cultivation)	
Corn steep liquor	30 g
Yeast extract	15 g
Ammonium phosphate Monobasic	3 g
Glucose (charge after sterilization) distilled water, q.s. to 1000 ml pH as is: 4.6, adjust to pH 6.5 with 20% NaOH, distribute 50 ml to 250 ml Erlenmeyer flask sterilize 121°C for 20 minutes.	30 g

- 20 The resulting suspension was used in an inoculum for the propagation of spores on agar plates. Ten agar plates were prepared, each bearing a solid glucose/yeast extract/phosphate/agar growth medium having the composition set forth in Table 16:

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TABLE 16 - GYPA (Glucose/Yeast Extract/Phosphate Agar for Plates)	
Glucose (charge after sterilization)	10 g
Yeast extract	2.5 g
K ₂ HPO ₄	3 g
Agar distilled water, q.s. to 1000 ml adjust pH to 6.5 sterilize 121°C for 30 minutes	20 g

A 0.2 ml aliquot of the suspension was transferred onto the surface of each plate. The plates were incubated at 25°C for ten days, after which the spores from all the plates were harvested into a sterile cryogenic protective medium having the composition set forth in Table 17:

TABLE 17 - GYP/Glycerol (Glucose/Yeast Extract/ Phosphate/Glycerol medium for stock vials)	
Glucose (charge after sterilization)	10 g
Yeast extract	2.5 g
K ₂ HPO ₄	3 g
Glycerol	20 g
Distilled water, q.s. to 1000 mL Sterilize at 121°C for 30 minutes	

The resulting suspension was divided among twenty vials, with one ml being transferred to each vial. These vials constitute a master cell bank that can be drawn on to produce working cell banks for use in generation of inoculum for bioconversion of canrenone to 11 α -hydroxycanrenone. The vials comprising the master cell bank were stored in the vapor phase of a liquid nitrogen freezer at -130°C.

To begin preparation of a working cell bank, the spores from a single master cell bank vial were resuspended in a sterile growth medium (1 ml) having the composition set forth in Table 15. This suspension was divided into ten 0.2 ml aliquots and each aliquot used to inoculate an agar plate bearing a solid growth medium having the composition set forth in Table 16. These plates were incubated for ten days at 25°C. By the third day of incubation, the underside of the growth medium was brown-orange. At the end of the incubation there was heavy production of golden colored spores. The spores from each plate were harvested by the procedure described

hereinabove for the preparation of the master cell bank. A total of one hundred vials was prepared, each containing 1 ml of suspension. These vials constituted the working cell bank. The working cell bank vials were
5 also preserved by storage in the vapor phase of a liquid nitrogen freezer at -130°C .

Growth medium (50 ml) having the composition set forth in Table 15 was charged to a 250 ml Erlenmeyer flask. An aliquot (0.5 ml) of working cell suspension
10 was introduced into the flask and mixed with the growth medium. The inoculated mixture was incubated for 24 hours at 25°C to produce a primary seed culture having a percent packed mycelial volume of approximately 45%. Upon visual inspection the culture was found to comprise
15 pellet-like mycelia of 1 to 2 mm diameter; and upon microscopic observation it appeared as a pure culture.

Cultivation of a secondary seed culture was initiated by introducing a growth medium having the composition set forth in Table 15 into a 2.8 L Fernbach
20 flask, and inoculating the medium with a portion (10 ml) of the primary seed culture of this example, the preparation of which was as described above. The inoculated mixture was incubated at 25°C for 24 hours on a rotating shaker (200 rpm, 5 cm displacement). At the
25 end of the incubation, the culture exhibited the same properties as described above for the primary seed culture, and was suitable for use in a transformation fermentation in which canrenone was bioconverted to 11α -hydroxycanrenone.

30 Transformation was conducted in a Braun E Biostat fermenter configured as follows:

Capacity:	15 liters with round bottom
Height:	53 cm
Diameter:	20 cm
35 H/D:	2.65
Impellers:	7.46 cm diameter, six paddles 2.2 x 1.4 cm each

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- Impeller spacing: 65.5, 14.5 and 25.5 cm from bottom of tank
 Baffles: four 1.9 x 48 cm
 Sparger: 10.1 cm diameter, 21 holes ~1 mm diameter
 Temperature control: provided by means of an external vessel jacket

Canrenone at a concentration of 20 g/L was suspended in deionized water (4 L) and a portion (2 L) of growth medium having the composition set forth in Table 18 was added while the mixture in the fermenter was stirred at 300 rpm.

TABLE 18 (Growth medium for bioconversion culture in 10 L fermenter)		
	Quantity	Amount/L
glucose (charge after sterilization)	160 g	20 g
peptone	160 g	20 g
yeast extract	160 g	20 g
antifoam SAF471	4.0 ml	0.5 ml
Canrenone deionized water q.s. to 7.5L sterilize 121°C for 30 minutes	160 g	20 g

The resulting suspension was stirred for 15 minutes, after which the volume was brought up to 7.5 L with additional deionized water. At this point the pH of the suspension was adjusted from 5.2 to 6.5 by addition of 20% by weight NaOH solution, and the suspension was then sterilized by heating at 121°C for 30 minutes in the Braun E fermenter. The pH after sterilization was 6.3±0.2, and the final volume was 7.0 L. The sterilized suspension was inoculated with a portion (0.5 L) of the secondary seed culture of this example that has been prepared as described above, and the volume brought up to

8.0 L by addition of 50% sterile glucose solution. Fermentation was carried out at a temperature of 28°C until the PMV reached 50%, then lowered to 26°C, and further lowered to 24°C when PMV exceeded 50% in order to maintain a consistent PMV below about 60%. Air was introduced through the sparger at a rate of 0.5 vvm based on initial liquid volume and the pressure in the fermenter was maintained at 700 millibar gauge. Agitation began at 600 rpm and was increased stepwise to 1000 rpm as needed to keep the dissolved oxygen content above 30% by volume. Glucose concentration was monitored. After the initial high glucose concentration fell below 1% due to consumption by the fermentation reaction, supplemental glucose was provided via a 50% by weight sterile glucose solution to maintain the concentration in the 0.05% to 1% range throughout the remainder of the batch cycle. Prior to inoculation the pH was 6.3 ± 0.2 . After the pH dropped to about 5.3 during the initial fermentation period, it was maintained in the range of 5.5 ± 0.2 for the remainder of the cycle by addition of ammonium hydroxide. Foam was controlled by adding a polyethylene glycol antifoam agent sold under the trade designation SAG 471 by OSI Specialties, Inc.

Growth of the culture took place primarily during the first 24 hours of the cycle, at which time the PMV was about 40%, the pH was about 5.6 and the dissolved oxygen content was about 50% by volume. Canrenone conversion began even as the culture was growing. Concentrations of canrenone and 11 α -hydroxycanrenone were monitored during the bioconversion by analyzing daily samples. Samples were extracted with hot ethyl acetate and the resulting sample solution analyzed by TLC and HPLC. The bioconversion was deemed complete when the residual canrenone concentration was about 10% of the initial concentration. The approximate conversion time was 110 to 130 hours.

When bioconversion was complete, mycelial biomass was separated from the broth by centrifugation. The supernatant was extracted with an equal volume of ethyl acetate, and the aqueous layer discarded. The mycelial fraction was resuspended in ethyl acetate using approximately 65 volumes per g canrenone charged to the fermentation reactor. The mycelial suspension was refluxed for one hour under agitation, cooled to about 20°C, and filtered on a Buchner funnel. The mycelial filter cake was washed twice with 5 volumes of ethyl acetate per g of canrenone charged to the fermenter, and then washed with deionized water (1 L) to displace the residual ethyl acetate. The aqueous extract, rich solvent, solvent washing and water washing were combined. The remaining exhausted mycelial cake was either discarded or extracted again, depending on analysis for residual steroids therein. The combined liquid phases were allowed to settle for two hours. Thereafter, the aqueous phase was separated and discarded, and the organic phase concentrated under vacuum until the residual volume was approximately 500 ml. The still bottle was then cooled to about 15°C with slow agitation for about one hour. The crystalline product was recovered by filtration, and washed with chilled ethyl acetate (100 ml). Solvent was removed from the crystals by evaporation, and the crystalline product dried under vacuum at 50°C.

Example 5

Lyophilized spores of Aspergillus ochraceus ATCC 18500 were suspended in a corn steep liquor growth medium (2 ml) as described in Example 4. Ten agar plates were prepared, also in the manner of Example 4. The plates were incubated and harvested as described in Example 4 to provide a master cell bank. The vials